METABOLISM AND NUTRITION

Effect of Pelleting Temperature on the Activity of Different Enzymes

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ABSTRACT The effects of different pelleting temperatures on the activity of cellulase, bacterial amylase, fungal amylase, and pentosanase were tested. Samples of a commercial barley-wheat-soybean diet containing different enzyme preparations were pelleted at 60, 70, 80, 90, and 100 C (pellet temperature measured at the die outlet) through a die containing holes 2.5 mm in diameter. Enzymatic analyses were conducted on either soluble substrates or by measuring the ability of the tested enzymes to decrease the viscosity of the diet. Measurements made on soluble substrates suggest that cellulase, fungal amylase, and pentosanase maintained activity when being pelleted at temperatures up to 80 C and bacterial amylase maintained activity at temperatures up to 90 C. Pentosanase and amylases showed little or no effect on the viscosity of the diet. Cellulase addition decreased the viscosity at all temperature levels, even after being pelleted at 90 and 100 C ($P < 0.05$). No cellulolytic activity was detected on the soluble substrate after these pelleting temperatures. Measurements on a soluble substrate might therefore not always reflect the true stability of a preparation because the ability of a carbohydrase to decrease the viscosity of the digesta is important to its effect in the gastrointestinal tract.

Measurements on soluble substrates suggest that cellulase, fungal amylase, and pentosanase can be pelleted at temperatures up to at least 80 C and bacterial amylase up to 90 C without a considerable loss in analyzed activity.

(*Key words*: pelleting temperature, enzyme thermostability, viscosity, radial diffusion)

1996 Poultry Science 75:357–361

INTRODUCTION

Recent studies have shown that enzyme supplementation has the potential to improve the nutritive value of feedstuffs for monogastric animals (Graham *et al.*, 1988; Annison, 1992; Wenk *et al.*, 1993). To reach their potential in improving the nutritive value of feedstuffs, enzymes have to be biologically active when reaching the gastrointestinal tract. The structure of an enzyme is critical to its activity. The structure of enzymes can be altered by exposure to heat, extremes of pH, or certain organic solvents such that activity can be decreased or completely abolished.

The temperature of feed is increased during the pelleting process by steam addition in the conditioner and by friction when being pressed through the die. Depending on the pelleting system and the conditions used during the process, the feed reaches temperatures between 60 and 90 C prior to entering the cooling system. As concern over accidental transmission of pathogens via contaminated feed increases, processing conditions have become more aggressive (Lyons and Walsh, 1993). The pelleting process can, therefore, lead to losses in activity of feed-borne and added enzymes (Rexen, 1981; Jongbloed and Kemme, 1990). Controversy exists to the extent of those losses. The losses appear to be affected by the type of enzyme preparation, the methods of assessing pelleting temperature, and enzyme recovery (Gadient *et al.*, 1993). This study was conducted to assess the stability of cellulase, pentosanase, bacterial amylase, and fungal amylase following pelleting at different temperatures.

MATERIALS AND METHODS

Pelleting

Samples of a commercial wheat-barley-soybean diet (Tables 1 and 2) containing no enzyme (control), cellulase (27,400 units/kg), pentosanase (3,350 units/kg), bacterial amylase (365,000 units/kg), and fungal amylase[1] (715,000 units/kg) were pelleted at different temperatures in a laboratory pelleting press.[2] Enzyme activities were determined using the methods provided by the manufacturer. The pelleting press was equipped with a die containing 2.5-mm holes. The steam load added to the conditioner was adjusted in a manner such that the pellet temperature

Received for publication February 24, 1995.
Accepted for publication October 25, 1995.
[1]All enzymes provided by Alltech Inc., Nicholasville, KY 40356.
[2]Modell DFPL 180.24,4, Buehler AG, Uzwil, Switzerland.

TABLE 1. Composition of the diet

Ingredients	Amount
	(kg/ton)
Barley	480.0
Wheat	355.0
Potato protein	40.0
Soybean meal	50.0
Meat-bone meal	22.0
L-Lysine-HCl	5.4
Calcium carbonate	8.6
Sodium chloride	6.0
Premix 30	8.0
Celite	25.0

TABLE 2. Particle size distribution of the feed before pelleting

Particle size	Amount
(mm)	(%)
<0.25	27.1
0.25 to 0.50	31.3
0.50 to 1.00	32.0
1.00 to 1.40	6.8
1.40 to 1.00	1.9
2.00 to 3.55	0.5
>3.55	0.4

reached 60, 70, 80, 90, and 100 C (± 1 C) at the die outlet. Conditioning time was approximately 15 s. The temperature in the conditioner was controlled by a thermostat and the pelleting temperature was determined with an infrared heat tracer.[3] The feeds were pelleted in batches of 20 kg. Three subsamples (~ 200 g) of each treatment were removed, combined, and stored at room temperature until analysis.

Analysis

Pellet hardness was determined with a spring hardness tester and its durability was measured by the Quick-test.[4] The pelleting quality was only determined on the control diet, as additives were thought to have little or no effect on pellet quality in the quantities applied. Pellet hardness was measured on 10 pellets per treatment and durability was evaluated in a single run.

The enzymatic activities of cellulase and amylases were determined by radial diffusion of enzyme into a substrate-containing gel (Walsh *et al.*, 1995). The enzyme was extracted from the feed prior to application on the gel. Five grams of feed ground through a 0.25-mm sieve was suspended in 45 mL of buffer (Table 3) on a orbital shaker for 1 h at 30 C. A sample of 10 mL was then removed and clarified by centrifugation at 2,000 × *g* for 10 min. The supernatant was then diluted and 0.2 mL were placed in a well (10 mm diameter) cut into the substrate containing gel (5 mm thick). The gel was incubated for 16 h, cooled to room temperature for 1 h, stained for 1 h, and destained for 5 min. The diameters of the clearing zones were then measured and compared to the zones of a standard curve. The standard curve was prepared by adding known amounts of the enzyme to be tested to the control feed. A graph showing well diameters vs concentrations was then plotted and results were read from the graph.

The assay conditions for each enzyme are shown in Table 3. Analyses for cellulase and fungal amylase were conducted at their pH optima. However, analysis for bacterial amylase had to be conducted below its pH optima (6.5) because feed-borne enzymes interfered with the analytical procedure close to neutrality.

Diffusion rate of feed-borne pentosanase made it impossible to measure the activity of the added pentosanase by radial diffusion. Pentosanase activity was determined on 4-0-Methy-D-glucorono-D-xylan dyed with Remazol Brilliant Blue.[5] Two milliliters of enzyme that had been extracted from the feed as described above were incubated in 1 mL of a solution containing 0.5% substrate prepared in 0.1 *M* phosphate buffer (pH 6.0) for 3 h at 50 C. The remaining unhydrolyzed substrate was then precipitated with 5 mL of ethanol containing 0.6% of HCl. The samples were then centrifuged for 10 min at 2,000 × *g* and the released dye in the solution was measured colorimetrically at 585 nm. The standard curve was established by adding known amounts of enzyme (12.5, 25, 50, 75, 100, or 125% of amount added to the test feed) to the control diet. All samples were extracted in triplicate and analyzed in duplicate. Viscosity was determined in a cone/plate viscometer[6] as described by Vucic-Vranjes and Wenk (1995).

Statistical Analysis

Differences between means were tested using Student's *t* test at a significance level of $P < 0.05$.

RESULTS AND DISCUSSION

The effect of different pelleting temperatures on pellet quality is shown in Table 4. Increasing pelleting temperature increased pellet hardness and improved pellet durability until about 80 C.

Figure 1 shows the activity of the tested enzymes (percentage of initial activity) determined on a soluble substrate after being pelleted at different temperatures. The hydrothermal stability varied among the different enzymes. Cellulase, pentosanase, and fungal amylase were stable up to pelleting temperatures of 80 C but lost more than 90% of the tested activity after being pelleted at 90 C ($P < 0.05$). Bacterial amylase was more stable. Sixty percent of the tested enzyme activity was preserved after pelleting at 100 C. The improved stability of bacterial amylase over the other tested enzymes is in agreement with stability data provided by

[3]3M Electrical Products Division, Austin, TX 70726.
[4]Quick test CW Z1, Pellet-Abriebtester, Chem. Werke, Düsseldorf, Germany.
[5]Number 66960, Fluka Chemie AG, CH-9470 Buchs, Switzerland.
[6]Digital Display Model DV-II+, Brookfield Engineering Laboratories, Inc., Stoughton, MA 02072.

TABLE 3. Assay conditions for enzyme analysis by radial diffusion

Enzyme	Buffer	Substrate	Incubation temperature	Stain	Antistain
			(C)		
Cellulase	50 m*M* Naacetate buffer, pH 4.8	CMC[1]	50	0.3% Congo red[3]	1 *M* NaCl
Bacterial amylase	100 m*M* acetate buffer, pH 5.0	Soluble potato starch[2]	50	2% KI 0.2% I_2	1 *M* NaCl
Fungal amylase	100 m*M* Naacetate buffer, pH 4.8	Soluble potato starch[2]	50	2% KI 0.2% I_2	1 *M* NaCl

[1]Carboxymethylcellulose, Number C-5678, Sigma Chemical Co., St. Louis, MO 63178-9916.
[2]Number S-2630, Sigma Chemical Co., St. Louis, MO 63178-9916.
[3]Number C-6767, Sigma Chemical Co., St. Louis, MO 63178-9916.

the manufacturer, who determined their data after temperature exposure of the enzymes in different buffered solutions. However, inactivation in buffered solutions occurs at lower temperatures than inactivation during the pelleting process. This difference might be the result of different water contents that result in different heat conductivities. In addition, a protective effect on enzyme activity might also be exerted by feed components. Cowan and Rasmussen (1993) also determined different hydrothermal stability for different enzyme preparations. The stabilities of pentosanases determined in their experiments were similar to the values reported in this study. Gadient *et al.* (1993) reported that the activity of different carbohydrases tested was not affected to a great extent if a critical temperature of around 75 C was not exceeded during the hydrothermal process. Nunes (1993) reported that steam-pelleting higher than 60 C strongly reduced phytase activity. These lower values are not in disagreement with the results reported here, as these data suggest that differences exist among different enzyme preparations.

Viscosity increased in all treatments with increasing pelleting temperatures (Figure 2). This is in agreement with findings by Vukic-Vranjes and Wenk (1995), who determined an increase in the viscosity from 1.59 to 3.79 cP when barley was extruded. These authors used the same analytical method as was used in this study. They stated that this increase is the result of starch gelatinization and solubilization of fiber. The addition of cellulase reduced viscosity at all temperature levels from 60 C (10% reduction) to 100 C (18% reduction) when compared to the control ($P < 0.05$). The reduction in viscosity caused by cellulase addition was maximal at 90 C (22% reduction). Reduction of viscosity by the addition of cellulase is in agreement with findings by White *et al.* (1981), who reported decreased viscosity of the digesta when cellulase from *Trichoderma viride* had been added to a diet containing β-glucan. The cellulase used in this study was also derived from *Trichoderma viride*. Inborr and Bedford (1994), using viscosity measurements, reported a lower stability for β-glucanase derived from *Trichoderma longibrachiatum* than values reported for cellulase in this trial. The temperature at the die outlet in their trial was between 80 and 90 C. Differences in enzymes, a longer remaining time in the conditioner, and differences in the analytical methods might account for these variations. Viscosity reduction achieved by endolytic enzymes is responsible for the majority of the improvement seen in young chicks fed high-viscosity cereals and the relative effectiveness of various enzymes can be related to their viscosity reducing capability (Annison, 1992; Rotter *et al.*, 1989, 1990). Petterson (1989) reported that high viscosity decreases diffusion rates of solutes within the digesta, which can in turn have an adverse effect on the rate of enzymatic degradation in the gastrointestinal tract. Furthermore, increased viscosity at the site of absorption could physically hinder efficient nutrient uptake (Fengler and Marquardt, 1988). Cellulase decreased the viscosity even after being pelleted at 90 and 100 C. At these temperatures little or no activity could be detected by radial diffusion.

The following two hypotheses could explain this difference. First, the cellulase could have been active during and shortly after the pelleting process before it was completely inactivated. The delay in inactivity could lead to the observed decrease in viscosity. Second, differences could exist in the thermostability of the endolytic and exolytic enzyme complex. Enzymes used as feed additives are relatively crude preparations often containing many different activities (Lyons and Walsh, 1993). The cellulase used in this study is a mixture of enzymes that contain endolytic and exolytic activity. Viscosity reduction is achieved by endolytic activity, whereas the release of reducing sugars from a soluble substrate is the result of exolytic activity. The different

TABLE 4. Effect of pelleting temperature on pellet hardness and durability as determined with a spring hardness tester and the Quick test. Hardness values are means ± SD from 10 pellets

Temperature	Hardness	Durability loss
(C)	(kPa)	(%)
60	3.1 ± 0.96[a]	32.3
70	5.1 ± 0.63[b]	24.4
80	5.5 ± 1.10[b]	4.0
90	6.9 ± 1.17[c]	1.6
100	9.6 ± 2.11[d]	2.4

[a–d]Means ± SD with no common superscript differ significantly ($P < 0.05$).

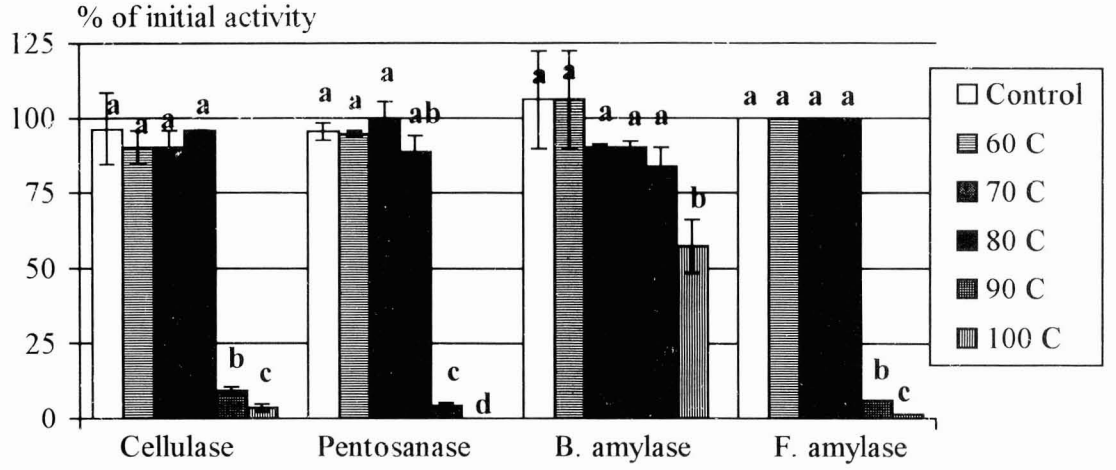

FIGURE 1. Amount of retained enzymatic activity in feed following pelleting at different temperatures. Assay methods as described earlier. Bars are means ± SD from three analyses and bars marked with different letters differ significantly $P < 0.05$. SD of F. amylase was negligible.

stabilities determined by the two analytical methods might be due to better thermostability of the endolytic in comparison to the exolytic enzyme complex.

Measurements on a soluble substrate might, therefore, not reflect the true stability of a preparation because the endolytic activity is more important for assessing the effect of an enzyme preparation in the gastrointestinal tract (Rotter *et al.*, 1990). Cowan and Rasmussen (1993) compared the activities of different pentosanases after various hydrothermal treatments on a native substrate and after extraction on a soluble substrate. These authors determined the same ranking in stability with both methods. However, they did not test whether treated enzyme with no activity on the soluble substrate was still active on the native substrate. The high hydrothermal stability noted with cellulase by viscosity measurement is in agreement with findings made in an *in vivo* trial by Vukic Vranjes *et al.* (1994), who detected a positive effect of added enzymes on broiler performance after extruding feed at 110 C.

The added pentosanase showed little ability to decrease the determined viscosity of the diet and its activities could not be determined by viscosity measurement. Many researchers have measured decreased viscosity with pentosanase addition (Bedford *et al.*, 1991; Campbell and Bedford, 1992). However, van der Klis (1993) stated that the effect of pentosanase on the viscosity of wheat-based diets greatly depended on the amount of wheat in the diet and the variety of wheat. The arabinoxylan in this diet was determined to be 1.94% using the method of Choct and Annison (1992). Assuming that all arabinoxylan in this diet is coming from the wheat (35% in the diet), the wheat would have contained 5.5% of arabinoxylan. This value would be rather low compared to values (5.0 to 8.0%) reported by Annison (1990), which could explain why pentosanase

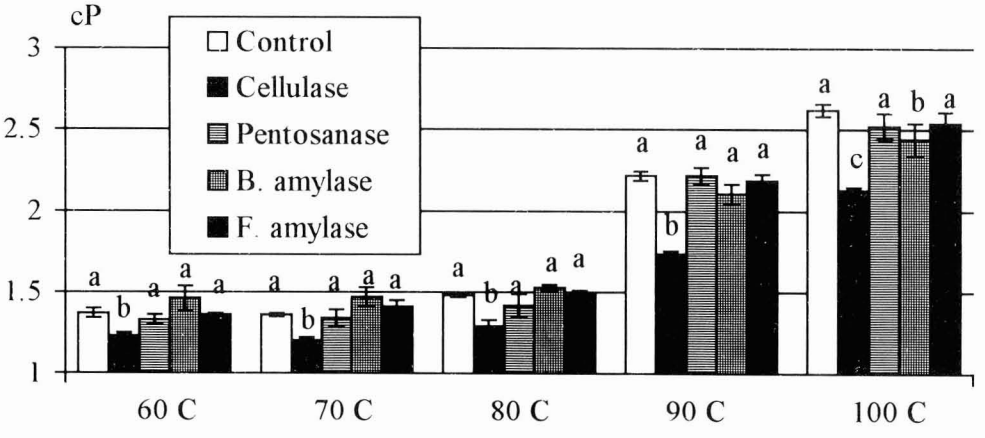

FIGURE 2. Effect of different enzymes on viscosity of feed after pelleting at various temperatures. Viscosity was measured using a cone plate viscometer. Feed was ground and incubated in phosphate buffer for (30 min., pH 6.0). The samples were then centrifuged and viscosity determined at 25 C and 60 rpm. Bars are means ± SD from three analyses and bars marked with different letters differ significantly $P < 0.05$.

had little effect on the viscosity. Bacterial amylase reduced viscosity only after being pelleted at 100 C ($P < 0.05$), which is in agreement with the high temperature stability measured by radial diffusion. Amylases might not have been effective at reducing the viscosity below pelleting temperatures of 90 C because the starch might not have exerted a measurable effect on the viscosity after exposure to these low pelleting temperatures.

These data suggest that the measured loss in enzymatic activity due to pelleting can vary with the analytical method used to assess the activity. Measurements on a soluble substrate suggest that cellulase, fungal amylase and pentosanase can be pelleted at temperatures up to at least 80 C and bacterial amylase up to 90 C without a considerable loss in activity. However, stability values for cellulase might even be higher as its addition decreased the viscosity of the diet even after being pelleted at 100 C.

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